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# Species-dependent effects of the phenolic herbicide ioxynil with potential thyroid hormone disrupting activity: modulation of its cellular uptake and activity by interaction with serum thyroid hormone-binding proteins

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**Abstract**

Ioxynil, a phenolic herbicide, is known to exert thyroid hormone (TH) disrupting activity by interfering with TH-binding to plasma proteins and a step of the cellular TH-signaling pathway in restricted animal species. However, comparative studies are still lacking on the TH disruption. We investigated the interaction of [$^{125}$I]ioxynil with serum proteins from rainbow trout, bullfrog, chicken, pig, rat, and mouse, using native polyacrylamide gel electrophoresis. Candidate ioxynil-binding proteins, which included lipoproteins, albumin and transthyretin (TTR), differed among the vertebrates tested. Rainbow trout and bullfrog tadpole serum had the lowest binding activity for ioxynil, whereas the eutherian serum had the highest binding activity. The cellular uptake of, and response to, ioxynil were suppressed by rat serum greater than by tadpole serum. The cellular uptake of [$^{125}$I]ioxynil competed strongly with phenols with a single ring, but not with THs. Our results suggested that ioxynil interferes with TH homeostasis in plasma and with a step of cellular TH-signaling pathway other than TH-uptake system, in a species-specific manner.

**Key words**: endocrine disruption; ioxynil; thyroid hormone; serum; transthyretin; uptake

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## Introduction

Humans and wildlife are exposed to many environmental chemicals through contaminated food, air, soil, sediments and surface water. Chemicals that are stable structurally accumulate in specific tissues of organisms via the food chain (Vallack et al., 1998). In some cases, these chemicals reach levels high enough to disrupt the development of the endocrine, immune, reproductive, and nervous systems of vertebrates, even if the concentration of these chemical are low or undetectable in environments where wildlife inhabit (Colborn and Clement, 1992; Boas et al., 2006).

The bioavailability and cellular actions of environmental chemicals in vertebrates are greatly influenced by how these chemicals are transported in the bloodstream (Nagel et al., 1998). Some chemicals, especially lipophilic chemicals, are transported as a protein-bound form in the bloodstream (Hjelmborg et al., 2008). Interestingly, plasma proteins that have a significant binding affinity for environmental chemicals include thyroid hormone (TH)-binding proteins (THBPs): lipoproteins and albumin for highly lipophilic chemicals, e.g., dioxins and polychlorinated biphenyls (Borlakoglu et al., 1990; Monteverdi and Di Giulio, 2000; Hjelmborg et al., 2008), and albumin and/or transthyretin (TTR) for relatively lipophilic chemicals that include synthetic estrogens (e.g., diethylstilbestrol), industrial and agricultural chemicals (e.g., tetrabromobisphenol A, triiodophenol and pentachlorophenol) (Sheehan and Young, 1979; Lans et al., 1993; Ishihara et al., 2003; Cao et al., 2011). These observations raise the possibility that environmental chemicals that bind to a THBP with high affinity may affect TH homeostasis in plasma (Lans et al., 1993; Ishihara et al., 2003; Cao et al., 2011).

Ioxynil (4-hydroxy-3,5-diiodobenzonitrile) is an iodine-containing phenolic compound that has been used as a herbicide. Its principal action is to inhibit photosynthesis at photosystem II by uncoupling oxidative phosphorylation in plants (Gromet-Elhanan, 1968). Ecotoxicity of ioxynil is greater in fish and amphibians than in birds and mammals (European Commission, 2004).

Ioxynil also acts as a thyroid system disrupting chemical (McKinlay et al., 2008; Morgado et al., 2009). This may be due to structural resemblance to THs: L-thyroxine ($T_4$) and 3,3',5-L-triiodothyronine ($T_3$). Previous studies indicated that ioxynil as well as diethylstilbestrol and tetrabromobisphenol A, with double rings, and triiodophenol and pentachlorophenol, with a single ring, interfered with TH binding to TTR *in vitro* (Ogilvie and Ramsden, 1988; Ishihara et al., 2003; Morgado et al., 2007). The binding affinity of TTRs for ioxynil was variable among species. In chicken and sea bream TTRs the binding affinity for ioxynil was comparable to that for $T_3$ (Ishihara et al., 2003;

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Morgado et al., 2007), whereas in human and amphibian TTRs it was 1–2 orders of magnitude lower than that for $T_3$ or $T_4$ (Ogilvie and Ramsden, 1988; Yamauchi et al., 2002; Ishihara et al., 2003). Furthermore, ioxynil affects the cellular TH-signaling pathway in amphibian culture systems (Shimada and Yamauchi, 2004; Sugiyama et al., 2005), although exact target sites of ioxynil within cells have not yet identified. In red blood cells (RBCs) from bullfrog tadpoles, TH receptor α gene is known to be up-regulated by TH (Murata and Yamauchi, 2004). Ioxynil inhibited this TH response at 0.2 μmol/L, but did not compete with TH binding to the receptor at the same concentration (Shimada and Yamauchi, 2004). Such a TH-antagonist activity of ioxynil was confirmed in a TH-responsive reporter gene assay using a *Xenopus laevis* cell line (Sugiyama et al., 2005). However, the extent to which ioxynil disrupts the extracellular and intracellular thyroid system among vertebrates is unclear.

THs are transported into target cells via various types of transporters: amino acid transporters and organic anion transporters (Hennemann et al., 2001), probably in a tissue-dependent manner. The amino acid transporters involved in TH uptake contain the transporters of large zwitterionic amino acid (System L) and the transporter linked to aromatic amino acid transporters (System T). In mammalian RBCs, System T is involved in TH uptake (Zhou et al., 1990). Recently the presence of similar TH uptake system was reported in the bullfrog tadpole RBCs (Shimada and Yamauchi, 2004). TH transport via these transporters may be a critical process that controls the intracellular TH levels and subsequent TH-dependent transcriptional activity of target genes. However, there have been no reports so far demonstrating that ioxynil interferes with the TH uptake systems.

The molecular and TH-binding properties of THBPs differ among vertebrate groups. In the rainbow trout, more than half of THs are bound to lipoproteins, predominantly high-density lipoprotein (Babin, 1992). In the bullfrog, major THBP is albumin although TTR also participates in TH binding in plasma of tadpoles from premetamorphic stages until the end of prometamorphic stage (Yamauchi et al., 1993). Birds and rodents have albumin and TTR as major THBPs in plasma (Schreiber and Richardson, 1997), even though thyroxine binding globulin (TBG) is restrictedly detected in serum from developing and aged rats (Savu et al., 1987, 1991). THBPs in large eutherians consist of albumin, TTR and TBG, among which TBG is a major THBP, because of its high affinity for THs (Robbins, 1996). TBG and TTR are binding proteins specific for THs whereas albumin binds a wide variety of lipophilic compounds including steroids and THs (Baker, 2002).

In the present study, we investigated the distribution of [$^{125}$I]-labeled ioxynil in serum from trout, bullfrogs, chickens, pigs, rats, and mice, to clarify which plasma proteins interact with ioxynil and if such interactions diverged in vertebrates. Furthermore, using tadpole and rat serum, which had low and high binding activities for ioxynil, respectively, we examined the effects of serum proteins on ioxynil uptake into, and TH-signaling disrupting activity within, cells *in vitro*.

## 1 Materials and methods

### 1.1 Reagents

Radiolabeled iodine ($Na^{125}I$; 629 GBq/mg as iodine) was purchased from PerkinElmer (Waltham, USA). Bisphenol A, $T_4$, $T_3$, pentachlorophenol, and 2-amino[2,2,1]heptane-2-carboxylic acid (BCH), were obtained from Sigma-Aldrich (St. Louis, USA). 2,4,6-Triiodophenol, 2,4,6-trichlorophenol, L-tryptophan, L-phenylalanine, L-leucine, sodium taurocholate were from Wako Pure Chemical Industries (Tokyo, Japan). Ioxynil (analytical standard) was purchased from Riedel-de Haën Fine Chemicals (Seelze, Germany). 4-Nonylphenol was from Nakarai Tesque (Kyoto, Japan). Bromosulfophthalein was purchased from MP Biomedicals (Irvine, USA). Sodium *p*-toluenesulfonchloramide trihydrate (chloramine-T) and chromatography-grade methanol were obtained from Kanto Chemical (Tokyo, Japan).

Phenol and phenolic compounds, were dissolved in dimethyl sulfoxide to concentrations of 2–10 mmol/L. Substrates of transporters were dissolved in phosphate-buffered saline (137 mmol/L NaCl, 2.7 mmol/L KCl, 1.5 mmol/L $KH_2PO_4$, 8.1 mmol/L $Na_2HPO_4$, pH 7.4) or HEPES-buffered saline (100 mmol/L NaCl, 2 mmol/L KCl, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 2 mmol/L $NaHCO_3$, 5.6 mmol/L glucose, 20 mmol/L HEPES, pH 7.5) to a concentration of 10–100 mmol/L. All chemicals were diluted with an appropriate buffer to give less than 0.4% (*V*/*V*) solvent. Control assays without test chemicals were performed with 0.4% (*V*/*V*) solvent. The solvent did not affect the competitive [$^{125}$I]ioxynil uptake assay and reporter gene assay.

### 1.2 Radiolabeling

Ioxynil was radiolabeled by iodine exchange reaction using the chloramine-T method (Ogilvie and Ramsden, 1988), with some modifications. An aliquot of the diluted ioxynil solution (660–1320 pmol) was mixed with $Na^{125}I$ (7.4 MBq), 280–560 pmol of unlabeled NaI, and 525 nmol of chloramine-T in 53 μL of 20 mmol/L sodium phosphate, pH 7.2. After incubating the mixture for 10 min, the radiolabeling was stopped by the addition of 16 μL of 4 mmol/L sodium disulfite. The reaction mixture was extracted with 300 μL of chloroform:acetic acid (99:1, *V*/*V*). The organic phase was collected and then evaporated with a test tube evaporator (type TVE-1000, EYELA, Tokyo, Japan). The evaporated organic phase was reconstituted in an appropriate volume of methanol:distilled water:acetic acid (74:25:1, *V*/*V*/*V*), and purified by high-performance liquid chromatography on a reverse-phase $C_{18}$ analytical column (Mightysil RP-18 GP, 250 mm × 4.6 mm, 5 μm particle diameter; Kanto Chemical, Tokyo, Japan), with an isocratic mobile phase (methanol:distilled water:acetic acid, 74:25:1, *V*/*V*/*V*) at a flow rate of 1.0 mL/min. The concentration of ioxynil was monitored by absorbance at 254 nm. The amount of purified ioxynil was estimated

from a calibration line of ioxynil at defined amounts. Both radiolabeled and unlabeled ioxynils were eluted at 6.2 min. The respective fractions were collected and radioactivity was measured in a γ-counter (Auto Well Gamma System ARC-380CL, Aloka, Tokyo, Japan). The specific activity of ioxynil was estimated from the amount of and the radioactivity of the purified ioxynil, which was 3.1–3.2 TBq/mmol (0.039–0.040 radioactive atom/ioxynil molecule), with a yield of 26%–57%. The purity of the isolated [$^{125}$I]ioxynil was assessed by thin-layer chromatography. Aliquots of the radiolabeled ioxynil were loaded on a plate (PE SIL G/UV, Whatman, Maidstone, UK) and run for 1.0–1.5 hr in chloroform/acetic acid (99/1, *V*/*V*) solvent followed by autoradiography. The purified ioxynil was stored in dimethyl sulfoxide at 4°C until required.

### 1.3 Biological materials

Rainbow trout (*Onchorhynchus mykiss*, male $n$ = 10, female $n$ = 10) blood was collected from sexually mature trout at the Fuji Trout Hatchery in the Shizuoka Prefectural Research Institute of Fishery, Fujinomiya, Shizuoka, Japan. Tadpoles in premetamorphic stages and adult bullfrogs (*Rana catesbeiana*, adult male $n$ = 3, adult female $n$ = 3, tadpoles $n$ = 50) were obtained from Saitama Amphibian Institute, Saitama, Japan, and their blood were collected, after anesthetized by immersion in or injection of 0.2% (*W*/*V*) ethyl 3-aminobenzoate methanesulfonic acid (Sigma-Aldrich, St. Louis, USA), as described previously (Yamauchi et al., 1993). Chicken (*Gallus gallus*, adult male $n$ = 3, adult female $n$ = 3, juvenile $n$ = 5) and pig (*Sus scrofa domesticus*, male $n$ = 3) blood were collected from mature animals at the Swine and Poultry Research Center in the Shizuoka Prefectural Research Institute of Animal Industry, Kikugawa, Shizuoka, Japan. Blood was also collected from 60-day-old chickens (male and female) at a local abattoir in Shizuoka, Japan. Sprague-Dawley rat (*Rattus norvegicus,* male $n$ = 4) and C3H/HeJ mouse (*Mus musculus*, male $n$ = 4) blood were kindly provided by Dr. T. Koike, Department of Biological Science, Faculty of Science, Shizuoka University. Tadpole RBCs were prepared as described previously (Yamauchi et al., 1989). Serum was separated from blood cells by centrifugation at 400 ×$g$ for 15 min at 4°C, and used immediately or stored at –20°C until required.

The care and treatment of animals used in this study were in accordance with the Guidelines for Proper Conduct of Animal Experiments, Japan.

### 1.4 Native polyacrylamide gel electrophoresis (PAGE)

Serum (100 μL) was incubated with 10 nmol/L [$^{125}$I]ioxynil ($2 \times 10^5$ dpm) for 1 hr at 4°C, and was analyzed by electrophoresis in a native 10% polyacrylamide gel in 0.05 mol/L Tris-HCl, pH 8.6, at 4°C (Richardson et al., 1994). To improve the separation of TTR from albumin, the gel buffer was pH 8.3 for mouse serum and pH 9.3 for pig serum. [$^{125}$I]Ioxynil-binding proteins were detected by a phosphorimager (Personal Molecular Imager FX$^{TM}$, Bio-Rad, Hercules, USA). An aliquot was used to detect proteins by staining with Coomassie Brilliant Blue R-250 (Sigma-Aldrich, St. Louis, USA). An additional 2 μL of the trout, bullfrog and chicken serum samples was analyzed to detect lipoproteins by staining with Sudan Black B (Blom et al., 2003). Albumin was identified as the most abundant protein in each serum sample. TTR was identified by its mobility relative to albumin (Thomas et al., 1990; Duan et al., 1995; Ishihara et al., 2003; Kato et al., 2009).

### 1.5 Uptake of [$^{125}$I]ioxynil into tadpole RBCs

[$^{125}$I]ioxynil ($5 \times 10^4$ dpm) was pre-incubated with tadpole in premetamorphic stages or rat serum (at a final concentration of 1%, 4%, and 10%) in 200 μL of HEPES-buffered saline for 30 min at 4°C. Uptake was initiated by mixing the [$^{125}$I]ioxynil solution (final concentration = 0.5 nmol/L) with 50 μL of the tadpole RBC suspension ($5.0 \times 10^6$ cell/tube) in HEPES-buffered saline at 25°C. In each experiment, RBCs were freshly prepared from three to four tadpoles at premetamorphic stages. In the competitive uptake assay, [$^{125}$I]ioxynil ($5 \times 10^4$ dpm) was mixed with tadpole RBCs ($5.0 \times 10^6$ cell/tube) in HEPES-buffered saline in the presence or absence of candidate competitors at 25°C. After 2 min, the cell-associated [$^{125}$I]ioxynil was separated from free [$^{125}$I]ioxynil by the oil-centrifugation method (Yamauchi et al., 1989). The tip of the polyethylene tube (0.4 μL microcentrifuge tube, Porex Bio Products, Petaluma, USA) containing the cell pellet was cut off. The radioactivity of the cell-associated [$^{125}$I]ioxynil was determined in a γ-counter.

Using tadpole RBCs, a system of saturable initial uptake for ioxynil was investigated, in the presence of potent competitors that were known as substrates of transporters (taurocholate and bromosulfophthalein for organic anion transporters, and BCH, tryptophan, leucine and phenylalanine for amino acid transporters), exogenous phenols with a single ring (triiodophenol, pentachlorophenol, ioxynil, trichlorophenol and nonylphenol), and exogenous (bisphenol A) and endogenous ($T_3$ and $T_4$) phenolic compounds with double rings.

### 1.6 $T_3$-responsive reporter gene assay

To investigate the effect of serum proteins on the $T_3$-antagonist activity of ioxynil in the reporter gene assay, either rat or bullfrog tadpole (in premetamorphic stages) serum was added to 70% Leibovitz's L-15 medium to a final concentration of 0%, 1%, or 4%. These mixtures were pre-incubated in the presence or absence of 1.0 μmol/L ioxynil for 24 hr at 25°C. Recombinant *Xenopus laevis* XL58-TRE-Luc cells, which express high levels of luciferase in a $T_3$-dependent manner (Sugiyama et al., 2005), were then incubated in the mixture, with or without 2 nmol/L $T_3$, for 24 hr. The cell lysate was assayed for firefly luciferase activity using the PicaGene Luminescence kit (Nippon Gene, Tokyo, Japan). The viability of the *X. laevis* XL58-TRE-Luc cells in the presence or absence of ioxynil was photometrically examined at 450 nm, as shown previously (Sugiyama et al., 2005). The addition of 1.0 μmol/L ioxynil did not affect the cell viability under our

experimental conditions.

### 1.7 Statistical analysis

The data presented are the mean ± SE ($n$ = 3), unless otherwise noted. Differences between groups were analyzed by Student's t test or a one-way analysis of variance, with the Scheffe test for multiple comparisons. $P < 0.05$ was considered statistically significant.

## 2 Results and discussion

### 2.1 Species-specific ioxynil-binding proteins in serum

The molecular species of ioxynil-binding proteins in serum differed among vertebrates. [$^{125}$I]Ioxynil-binding proteins in trout serum (lanes 1 and 2, and lanes 12 and 13, Fig. 1a) corresponded partially to lipoproteins (lanes 1 and 2, Fig. 1b). The major [$^{125}$I]ioxynil-binding proteins in serum from the tadpole and adult bullfrogs (lanes 3–5 and 14, Fig. 1a) corresponded to albumin and lipoproteins (lanes 3–5, Fig. 1b). More than half of input [$^{125}$I]ioxynil was detected as a free form in the trout and bullfrog serum.

In chicken serum, two distinct [$^{125}$I]ioxynil-binding proteins corresponding to TTR and albumin were detected (lanes 6–8, Fig. 1a). [$^{125}$I]ioxynil-binding was also detected as a slow-migrating band (lane 8, Fig. 1a) partially corresponding to lipoproteins (lane 8, Fig. 1b) in female chicken serum. Collection of the serum from female chickens laying eggs may have contributed to the sex-specific differences detected in ioxynil-binding proteins.

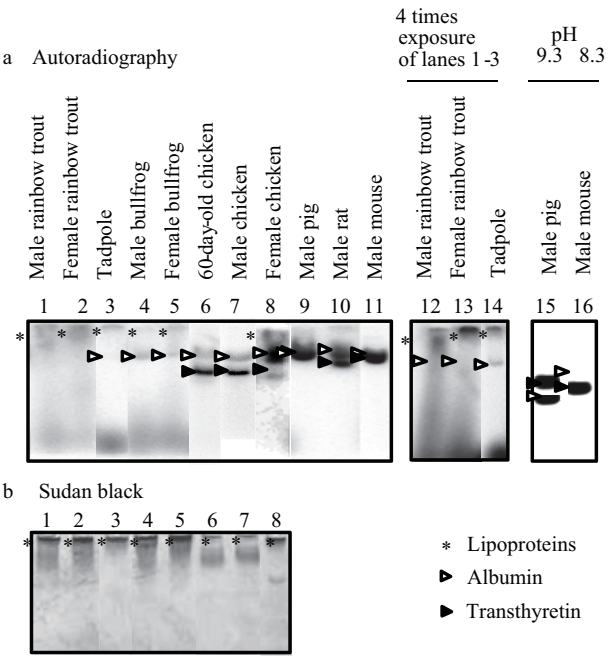


**Fig. 1** Native polyacrylamide gel electrophoresis (PAGE) of [$^{125}$I]ioxynil binding proteins in serum from rainbow trouts, bullfrogs, chickens, pigs, rats, and mice. (a) Binding of [$^{125}$I]ioxynil ($1 \times 10^4$ dpm) to serum proteins (lanes 12–14, exposure length four times that of lanes 1–3). Proteins were separated by native PAGE at pH 8.6 (lanes 1–14), pH 9.3 (lane 15), or pH 8.3 (lane 16). (b) Lipoproteins in the serum detected with Sudan Black (lanes 1–8). Positions of lipoproteins, albumin and transthyretin are marked. Experiments were repeated three times, using serum from different individuals of each species.

The major [$^{125}$I]ioxynil-binding protein (pH 8.6) in the pig and mouse serum corresponded somewhat to albumin (lanes 9 and 11, respectively, Fig. 1a). However, native PAGE of pig serum at pH 9.3 resulted in two distinct bands (lane 15, Fig. 1a) that corresponded to albumin (fast-migrating band) and TTR (slow-migrating band). Native PAGE of mouse serum at pH 8.3 separated the major [$^{125}$I]ioxynil-binding protein from albumin, which did not interact with [$^{125}$I]ioxynil (lane 16, Fig. 1a). This result was similar to that for rat serum (native PAGE at pH 8.6; lane 10, Fig. 1a).

TTR was the major ioxynil-binding protein in serum from juvenile and adult male chickens, male pigs and male rats, and was sole ioxynil-binding protein in mouse serum. Although sea bream and bullfrog TTRs have been previously shown to bind ioxynil (Morgado et al., 2007; Ishihara et al., 2003), we did not detect ioxynil binding to TTRs in trout and tadpole serum. This may in part be because of different assays used in each study. Halogenated phenols resemble the phenol moiety of THs in structure with halogen in either *ortho* position or both *ortho* positions, with respect to the hydroxygroup. The iodine position of ioxynil (3 and 5 positions) is the same as that of the $T_4$ phenolic ring. In male chickens and rats, TTR was not only the major ioxynil-binding protein but also the predominant THBP (Thomas et al., 1990), suggesting the interference of ioxynil with TH homeostasis in plasma. Such TTR-mediated adverse effects on plasma TH homeostasis may not be applicable to large eutherians, such as pigs and humans, because TBG, which has a higher affinity for THs than TTR (Janssen et al., 2002), is the major THBP, but not a ioxynil-binding protein, in their plasma.

### 2.2 Effects of serum and chemicals on [$^{125}$I]ioxynil uptake into tadpole RBCs

We selected tadpole and rat serum, which had the lowest and the highest ioxynil-binding activities, respectively, also selected tadpole RBCs, which have well characterized TH uptake system (Yamauchi et al., 1989; Shimada and Yamauchi, 2004) and are TH-responsive via TH receptor α gene (Murata and Yamauchi, 2004), and investigated the effect of the serum (0%, 1%, 4%, or 10%) on the uptake of [$^{125}$I]ioxynil into tadpole RBCs (Fig. 2a). [$^{125}$I]ioxynil associated with RBCs reached a plateau after 2 min, with approximately 10% of [$^{125}$I]ioxynil added to the media (control; data not shown). Rat serum inhibited [$^{125}$I]ioxynil uptake into the cells in a concentration-dependent manner, to a greater extent than tadpole serum ($P < 0.01$). Rat serum has high [$^{125}$I]ioxynil binding capacity, so there is less free [$^{125}$I]ioxynil available to be taken up into cells. Whereas tadpole serum has reduced [$^{125}$I]ioxynil binding capacity, resulting in a higher concentration of free [$^{125}$I]ioxynil which is available to be taken up into cells. This result suggests that, on exposure to ioxynil, the concentration of unbound ioxynil in tadpole blood is likely to be high, which may result in increased uptake.

THs are transported into cells by specific transporters such as amino acid transporters and organic anion transporters (Hennemann et al., 2001). To clarify whether

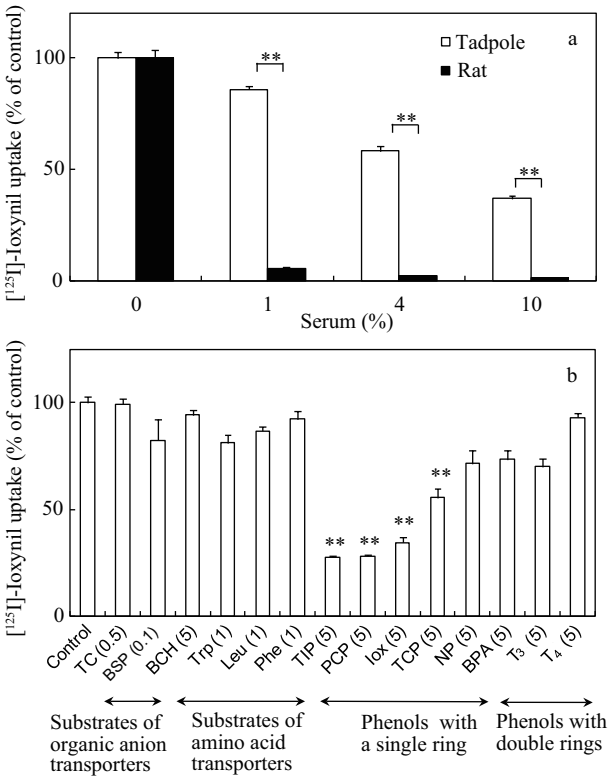


**Fig. 2** Cellular uptake of [$^{125}$I]ioxynil. Saturable initial uptake of [$^{125}$I]ioxynil into tadpole red blood cells was examined in HEPES-buffered saline (a) in the presence or absence (0%, control) of tadpole or rat serum, and (b) in the presence or absence (control) of chemicals. Chemicals used were: taurocholate (TC) and bromosulfophthalein (BSP), 2-amino[2,2,1]heptane-2-carboxylic acid (BCH), tryptophan (Trp), leucine (Leu), phenylalanine (Phe), triiodophenol (TIP), pentachlorophenol (PCP), ioxynil (Iox), trichlorophenol (TCP), nonylphenol (NP), bisphenol A (BPA), triiodothyronine ($T_3$), and thyroxine ($T_4$). Concentrations (mmol/L for transporter substrates and μmol/L for the other chemicals) are indicated in parenthesis. Experiments were repeated three times. $^{**}P < 0.01$.

cellular ioxynil uptake is mediated by some of these transporters, the effects of substrates and inhibitors for transporters were tested on [$^{125}$I]ioxynil uptake into tadpole RBCs. Of the six substrates for the organic anion (taurocholate and bromosulfophthalein: concentration = 0.1–0.5 mmol/L) and amino acid (BCH, tryptophan, leucine and phenylalanine: concentration = 1–5 mmol/L) transporters, none inhibited [$^{125}$I]ioxynil uptake significantly (Fig. 2b). In contrast, four (triiodophenol, pentachlorophenol, ioxynil, trichlorophenol: concentration = 5 μmol/L) of the five phenols with a single ring inhibited [$^{125}$I]ioxynil uptake significantly ($P < 0.01$). Two (bisphenol A and $T_3$: concentration = 5 μmol/L) of the three phenolic compounds with double rings tended to inhibit slightly [$^{125}$I]ioxynil uptake but this effect was not statistically significant. This strongly suggests that phenols with a single ring, including ioxynil, are transported into RBCs by an uptake system that is distinct from transporters for organic anions, amino acids, and THs. Thus, ioxynil may not compete cellular TH upake. THs are transported by several uptake systems: the amino acid transporters for large zwitterionic amino acids (System L) and the aromatic amino acids (System T), and several subtypes of organic anion transporters (Hennemann et al., 2001). In tadpole RBCs, System T transporter is involved in TH uptake (Shimada and Yamauchi, 2004).

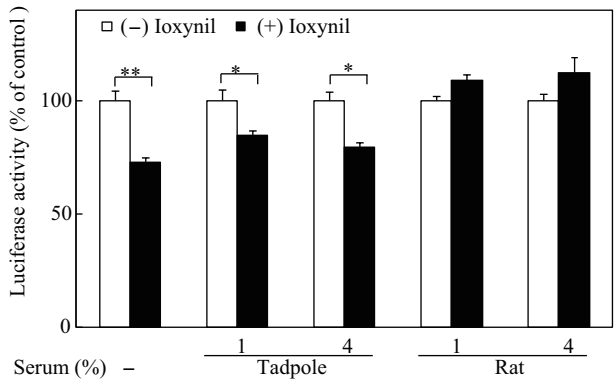


**Fig. 3** Effect of serum on $T_3$-antagonist activity of ioxynil in the $T_3$-responsive reporter gene assay. Recombinant XL58-TRE-Luc cells were treated with 2.0 nmol/L $T_3$, in the presence or absence (control) of 1.0 μmo/L of ioxynil. These experiments were performed in serum-free 70% Leibovitz's L-15 medium, 70% Leibovitz's L-15 medium containing bullfrog tadpole and rat serum (1% and 4%). The vertical axes represent the luciferase activity as a percent of control. Experiments were repeated three times. $^{*}P < 0.05$ and $^{**}P < 0.01$.

### 2.3 Effects of serum on $T_3$-antagonist activity of ioxynil in a recombinant *X. laevis* cell line

We investigated the effects of tadpole and rat serum (1% and 4%) on $T_3$-antagonist activity of ioxynil using a $T_3$-responsive reporter gene assay (Fig. 3). In our reporter gene system using *X. laevis* XL58-TRE-Luc cells, ioxynil (at 0.2–1.0 μmol/L) inhibited the $T_3$-dependently induced luciferase activity (Sugiyama et al., 2005). The luciferase activity was inhibited to 76% by 1.0 μmol/L ioxynil in the absence of serum. Tadpole serum weakened slightly but rat serum abolished completely the $T_3$-antagonist activity of ioxynil even at 1% concentration. No effect of ioxynil on the basal expression levels of luciferase was observed in all groups without $T_3$ (data not shown). The cellular actions of ioxynil, including the interference of the TH signaling pathway within cells, may be modulated by serum binding proteins, depending on their binding affinity and capacity for ioxynil. This could be one of the reasons for greater ecotoxicity of ioxynil in fish and amphibians than in birds and mammals (European Commission, 2004).

## 3 Conclusions

Ioxynil is thought to elicit TH disrupting activity by at least two different mechanisms: the interference with TH homeostasis in plasma by displacing TH from THBPs and with cellular TH action by affecting a step of the TH signaling pathway other than TH uptake system. Our study demonstrated that the binding of ioxynil to serum proteins differed quantitatively and qualitatively in a species-specific manner. These differences may reflect an evolutionary change in plasma ioxynil-binding proteins. The amount of ioxynil bound to serum proteins was less in lower vertebrates than in higher vertebrates, depending on the extent to which albumin and TTR bound ioxynil. Therefore, in rat ioxynil may largely interfere with TH

homeostasis in plasma, with little direct effect within cells. In contrast, in tadpole ioxynil may be easily transported into cells and exert cellular action rather than the interference with TH homeostasis at plasma level.

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